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Katharine Torrey  
kaytorrey@gmail.com

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Context-specific effects of vasotocin on social approach in the  
male common goldfish, *Carassius auratus*

An Honors Paper for the Program of Neuroscience

By Katharine Rebecca Torrey

Bowdoin College, 2019

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## ABSTRACT

The peptide vasotocin (VT) and its mammalian homologue, vasopressin (VP), produce effects on social behavior that are highly species- and context-specific. In male goldfish, VT works through a hindbrain circuit to inhibit approach to other males, but little is known about its function in the forebrain or in social contexts related to courtship and reproduction. We recently sequenced two genes for V1a-like receptors (VTR) in the goldfish brain, one that encodes for a fully-functioning canonical receptor and one that encodes for a non-functional truncated receptor. The current study is an anatomical and behavioral investigation of whether social context may alter expression of these receptor types and thus, potentially, behavioral responses to VT. We used western blotting and immunohistochemistry with custom anti-VTR antibodies to characterize the distribution of VTR throughout the forebrain and the hindbrain. Western blot results showed bands close to the predicted sizes for truncated and canonical VTR constructs, suggesting that both genes are translated into protein in the brain, but the presence of additional bands suggested potential nonspecific binding. Immunohistochemistry data revealed VTR signal throughout the brain in regions associated with social behavior, including the telencephalon, preoptic area, optic tectum, dorsal motor vagus, and area postrema, with overlapping distribution patterns of canonical-specific and combined canonical/truncated signal. VTR signal also colocalized with a marker for radial glial cells, particularly in the telencephalon. We additionally examined whether visual and olfactory context alters behavioral responsiveness to VT, potentially by altering the expression of one or both receptors. Behavioral tests suggested that VT inhibits approach to males, but its effect on response to females in reproductive contexts is still undetermined, likely due to interference from a stress response during testing. Further characterization of VTR throughout the brain will clarify how social context might alter VT signaling through context-dependent modulation of its receptors. Additionally, future work should examine the behavioral consequences of such modulation by further studying whether VT's effect on social approach behavior depends on context.

## INTRODUCTION

Social behavior is regulated through a complex combination of environmental and physiological conditions that influence how animals sense and process social stimuli to produce appropriate behavioral responses. Differences in social cues and context result in a great variety of adaptive behaviors, some of which, including communication, mating, aggression, and parental care, are characteristic of almost all animals (Goodson and Kabelik, 2009). Social stimuli are primarily processed in the social behavior network (SBN), a group of brain regions that were first identified in mammals but have now been described across vertebrates in a conserved manner (O'Connell and Hofmann, 2011). The SBN is closely linked to the reward system and is comprised of six core nodes, each of which is involved in more than one type of social behavior: the medial extended amygdala, preoptic area, lateral septum, ventromedial hypothalamus, anterior hypothalamus, and midbrain periaqueductal/central gray (O'Connell and Hofmann, 2011). Importantly, it is the *pattern* of activity across this network—activated by a combination of external stimuli and internal conditions—that is essential to producing variability in social behavioral responses (Goodson and Kabelik, 2009). Neuropeptides such as vasotocin (VT) bind to receptors across multiple SBN nodes and influence how vertebrates integrate and respond to environmental cues (Albers, 2015).

### ***Vasotocin (VT) and vasopressin (VP)***

The SBN is rich with receptors for VT and its mammalian homologue, VP, which are important neuropeptides for a wide range of social behaviors (Albers, 2015). VT and VP are nine amino acids long and are differentiated only at the third position, where they contain an

isoleucine or a phenylalanine respectively (Goodson and Bass, 2001). The conserved evolutionary history of this peptide family is exhibited by similarities in cell populations, fiber distributions, receptor structure, and social behavioral effects (Goodson and Thompson, 2010; Thompson and Walton, 2004; Goodson and Bass, 2001).

Across vertebrates, populations of VT/VP magnocellular and parvocellular neurons exist in the preoptic area and anterior hypothalamus, which extend axons to the pituitary, midbrain tegmentum, and hindbrain (Albers, 2015; Goodson and Bass, 2001; Goodson and Kabelik, 2009; Goodson and Thompson, 2010; Thompson and Walton, 2009). Tetrapod vertebrates have also developed additional VT/VP cell populations in the lateral and medial amygdala and bed nucleus of the stria terminalis, which project to additional SBN regions (Goodson and Bass, 2001). Thus, there are multiple, central circuits through which these molecules can influence a variety of social responses.

In mammals, VP binds to four G-protein coupled receptors (V1a, V1b, V2, and OT), with V1a receptors mediating many of the peptide's effects on social responses (Albers, 2015). Analogous receptors for VP and VT have been identified in other vertebrate groups, each with characteristically similar structures consisting of seven transmembrane domains (Lema, 2010). VT/VP systems are well conserved across vertebrates with broad similarities in cell populations in the SBN, yielding functionally-related effects on social behavior. However, VT/VP systems can also vary widely in terms of VT/VP levels, terminal field locations, and receptor distributions (Goodson and Kabelik, 2009). Neuropeptide receptors, in particular, can vary in quantity, location, gene sequence, and binding sensitivity, resulting in varied patterns of SBN activation (Albers, 2015; Goodson and Thompson, 2010). In fact, receptors in the VT/VP family have been shown to vary more between species than do other



neuropeptide receptors (Insel and Shapiro, 1992). In a review of work on VT/VP functioning in vertebrates, Goodson and Bass (2001) found evidence for distinctive expressions of V1a-like receptors in all species studied. Alongside differences in receptor distribution, differences in receptor construct can also produce behavioral variation. Zhu and Wess (1998) provide evidence for a truncated version of the V2 receptor, which can dimerize with the full-length, canonical V2 receptor to decrease sensitivity to VP. While there is a lack of published evidence for such a phenomenon in other VT/VP receptors, including the V1a receptor, it is highly possible that they may undergo similar truncation and dimerization processes.

Differences in VT/VP neuron populations, projections, receptor distributions, and receptor constructs all contribute to the formation of a great diversity of SBN neural activity patterns, resulting in wide behavioral variation and suggesting that this system has evolved to help animals adjust to rapidly changing social environments (Goodson and Kabelik, 2009). Additionally, the specific pattern of SBN activation, as a result of other external and internal conditions, can alter VT/VP functioning (Goodson and Kabelik, 2009). As will be explored in the following three sections, it is likely that factors including species, sex, and context can alter VT/VP mechanisms to produce specific behavioral modulation.

### ***Species-specific effects of VT***

While the basic physiological properties of the VT/VP circuit are similar among vertebrates, the binding of these peptides to receptors distributed across SBN nodes are associated with diverse behavioral outputs, including sociality, courtship, aggression, pair-bonding, and parental behavior. Goodson and Bass (2001) reviewed available evidence of VT/VP's modulation of behavior and concluded that its effects are diverse and often

opposite, even between closely-related species. For instance, VT is associated with a decrease in aggression in the pupfish and an increase in aggression in the damselfish (Lema and Nevitt, 2004; Santangelo and Bass, 2006). Similarly, VT is related to an increase in courtship behaviors in the peacock blenny and a decrease in general sociability in zebrafish and goldfish (Carneiro et al., 2003; Thompson and Walton, 2004; Lindeyer et al., 2015). VP is also related to human social behavior. Men treated with intranasal VP showed a different pattern of prefrontal-amygdala fMRI connectivity to pictures of negatively-valenced emotional faces, suggesting that VP is related to the processing of fear and social stimuli (Zink et al., 2010). Furthermore, Thompson et al. (2006) showed that VP can actually promote antisocial behavioral responses in men. Men given VP intranasally rated strangers as less friendly and less approachable than did men administered placebo. It is important to examine how and why VT/VP acts across vertebrates to yield such distinct but related behavioral consequences.

The prairie vole and the montane vole are two related but separate species that provide evidence that VT impacts social behavior differently across even closely related species by activating a different pattern of V1a receptors within the SBN. Prairie voles, which are monogamous animals, have greater expression of V1a receptors in the ventral forebrain, particularly the ventral pallidum and substantia innominata, than do nonmonogamous montane voles (Young et al., 1999; Pitkow et al., 2001). Young et al. (1999) showed that VP treatment promotes affiliative behaviors in prairie voles, while it has no such effect in montane voles. Furthermore, Pitkow et al. (2001) showed that pair bonding and affiliative behaviors can be increased in prairie voles by using viral vector gene transfer to raise the expression of V1a receptors in the ventral pallidum. Together, these results

demonstrate the importance of mechanistic differences in VT/VP receptor systems to yield distinct social behaviors across species.

### ***Sex-specific effects of VT***

Even within the same species, VT/VP mechanisms are sexually dimorphic and can be mediated by sex hormones, which alter how these neuropeptides influence behavior in different sexes (Goodson and Bass, 2001). In male Syrian hamsters, VP binds to V1a receptors to increase aggressive actions, an effect that is blocked with a V1a receptor antagonist (Gutzler et al., 2010). However, Gutzler et al. (2010) showed that in female hamsters, treatment with the V1a receptor antagonist actually stimulated aggressive behaviors, while VP treatment decreased aggression, indicating that VP's modulation of aggressive behaviors in Syrian hamsters is sex-specific. Likewise, Godwin et al. (2000) used in situ hybridization to show that the number of magnocellular preoptic VT cells changes depending on the sex of the bluehead wrasse, which have three sex phenotypes consisting of females, non-aggressive males, and dominant males. They found that VT mRNA levels were higher in both types of males than in females, and that they were higher in dominant males than in non-aggressive males. Furthermore, they saw sharp elevations in VT mRNA levels during sex change, which coincided with increases in aggressive behaviors (Godwin, 2000). Thus, sex-specific variation in levels of VT neurons correlated with sex-specific changes in social behavior in bluehead wrasse (Godwin, 2000). In their review of VT/VP work, Goodson and Bass (2001) noted that while the majority of previous literature is focused on increased VT/VP effects in males rather than in females, this may not necessarily imply that these peptides are more linked to male behavior but rather that male-specific behaviors, such

as mate calling, have been more extensively studied. Sex-specific VT effects are relevant in humans, too. Thompson et al. (2006) showed that VP promoted antisocial facial motor patterns in males, whereas it had the opposite effect in females, promoting affiliative facial expressions in response to unfamiliar same-sex facial stimuli.

### ***Context-specific effects of VT***

Further evidence suggests that VT/VP can also act differently within the same sex and species to produce different behavioral responses depending on the context, which may be related to changes in environmental, seasonal, and social conditions. Context-specific changes in environmental conditions can alter the development, distribution, and activation of V1a receptors, which in turn impact VT/VP's modulation of behavior (Albers, 2015; Godwin and Thompson, 2012). Lema (2006) studied VT immunoreactivity responses to environmental changes in two separated populations, Big Spring and Amargosa River, of the same species of Death Valley pupfish, which have each adapted to live in different conditions: While Big Spring maintains a constant salinity and temperature throughout the seasons, the Amargosa River's salinity changes seasonally and temperatures fluctuate from day to night. They found that these two populations not only showed different VT immunoreactivity patterns, even when raised identically in lab, but also displayed a different relationship between VT neuron size and aggression. In Amargosa River pupfish, smaller magnocellular VT neurons were related to higher levels of aggression, while this was not true for Big Spring pupfish. These results provide evidence that VT mechanisms associated with behavioral regulation, even within the same species, can change depending on environmental context.

Seasonal changes, which occur alongside changes in sex steroid functioning, are also widely associated with variation in VT/VP cell populations, release, and receptor expression across vertebrates (reviewed in Goodson and Bass, 2001). For example, Ota et al. (1999) used in situ hybridization to measure mRNA expression for VT in male masu salmon five times over the course of a year. They found that elevations in VT mRNA levels fluctuated between months, with the highest levels in January. It is possible that as its circulating levels change by season, VT may take more or less time to produce similar behavioral effects at different times of the year.

In addition, changes in social contexts have been shown to alter VT/VP functioning in many species. Goodson et al. (2009) showed that in male violet-eared waxbills, blocking the V1a receptor with an antagonist decreased aggressive behaviors when the birds were fighting for mates but had no effect on aggression in the separate social context of a resident-intruder paradigm. The authors concluded that the presence of VT in different cell groups during these two social contexts may account for this difference by activating two distinct networks of SBN receptors (Goodson et al., 2009). Marler et al. (1999) provided further evidence that behavioral context can alter VT functioning by using immunocytochemistry to examine the distribution of VT immunoreactivity in brains of male cricket frogs exhibiting either “calling” or “noncalling” mating behavior. They found higher VT immunoreactivity in the nucleus accumbens in noncalling males, showing that different behavioral mating tendencies correlate with differences in VT functioning. Additionally, Cooper et al. (2005) used autoradiography to measure VP binding to V1a receptors in male Syrian hamsters following various socially agonistic experiences. They found that dominant hamsters showed more VP binding in the ventromedial hypothalamus after repeated victory experiences than

subordinate hamsters who had instead faced repeated social defeat experiences. This suggests that the social experience of winning or losing a conflict can influence the binding characteristics of VP, perhaps by changing the distribution of V1a receptors in particular locations and therefore altering how the hamsters subsequently respond to VP, which typically promotes aggression this species. Importantly, like seasonal changes, differences in social context are also related to fluctuations in circulating sex steroid levels, which may be linked to VT/VP functioning (reviewed in Goodson and Bass, 2001).

While these studies present initial evidence that differences in natural and social environments can alter VT/VP processes in vertebrates, many questions remain about the conditions and mechanisms behind such context-dependent modulation of VT/VP functioning (Albers, 2015; Goodson and Bass, 2001). The evolutionarily conserved nature of VT/VP systems allow for intriguing comparisons across vertebrates (Goodson and Thompson, 2010). Teleosts, including the goldfish, are useful models for studying behavioral modulation of neuropeptides because they exhibit complex and diverse social communication systems (Godwin and Thompson, 2012). As reviewed by Godwin and Thompson (2012), VT is related to many teleost behaviors including courtship, aggression, and sociality.

### ***VT system in the male goldfish***

Unlike in tetrapods, but as in other teleosts, VT in the goldfish is produced only in the preoptic area/hypothalamus (Thompson and Walton, 2009). Fibers from some of these VT neurons then project and terminate in regions in the hindbrain, including the dorsal motor vagus, medial nucleus of Cajal, and area postrema, and in the telencephalon, particularly the dorsal telencephalon (Thompson and Walton, 2004; Thompson and Walton, 2009). Preoptic

and hindbrain VT circuits are more conserved than forebrain circuits, with similar projections found in all vertebrates, including jawless fish (reviewed in Godwin and Thompson, 2012)—and, in goldfish, at least, those circuits are associated with VT's ability to promote social withdrawal (Thompson and Walton, 2004). In contrast, very little work has examined VT functioning in the goldfish forebrain.

VT in goldfish, as in all teleosts, binds to three types of G-protein coupled receptors (one V2-like receptor and two V1a-like receptors), each coded for by a different gene (Lema, 2010). These receptors are structurally related to mammalian VP receptors and are linked to a second messenger cascade producing effects through inositol phosphates (Goodson and Bass, 2001). Walton et al. (2010) showed that goldfish V1a-like receptor expression varied by season, particularly in the hindbrain, with highest levels during the mating season when VT exerts its effects on social withdrawal through actions in that circuit.

Recent unpublished work in the Thompson lab has identified a 3' truncated V1a receptor gene in goldfish, which lacks the 7<sup>th</sup> transmembrane domain necessary for VT binding (Andersen, 2017). Figure 1 shows the amino acid sequence for the full-length, fully-functioning V1a-like receptor transcribed by the canonical gene and the shortened, non-functional V1a-like receptor transcribed by the truncated gene. The predicted sizes for the canonical and truncated receptors are 45.44 kD and 26.69 kD respectively. It has yet to be determined whether both canonical and truncated forms of this receptor are actually translated in the goldfish brain, but initial western blot findings from Andersen (2017) using a custom anti-V1a-like receptor antibody suggested that they might. Given that dimerization occurs for V2 receptors (Zhu and Wess, 1998), it is possible that the truncated form of the V1a-like receptor can dimerize with the canonical form, decreasing VT binding in different

circuits within the brain. The expression of canonical, truncated, and dimerized V1a-like receptors in goldfish may even depend on the social context—determined by visual and olfactory cues—and thus yield context-dependent VT modulation of social behavior.

1	M	G	N	A	S	N	Q	T	T	A	<b>N</b>	<b>N</b>	<b>D</b>	<b>T</b>	<b>D</b>
<b>16</b>	<b>P</b>	<b>F</b>	<b>G</b>	<b>R</b>	<b>N</b>	<b>E</b>	<b>E</b>	<b>V</b>	<b>A</b>	<b>K</b>	M	E	I	T	V
31	L	S	V	T	F	L	V	X	V	M	G	N	L	C	V
46	L	L	A	M	H	N	T	K	K	K	S	S	R	M	H
61	L	F	I	K	H	L	S	L	A	D	L	V	V	A	F
76	F	Q	V	L	P	Q	L	C	W	E	I	T	F	R	F
91	Y	G	P	D	F	L	C	R	I	V	K	H	L	Q	V
106	L	G	M	F	A	S	T	Y	M	M	V	M	M	T	L
121	D	R	Y	I	A	I	C	H	P	L	K	T	L	Q	Q
136	P	T	K	R	A	Y	I	M	I	G	C	T	W	L	C
151	S	L	L	L	S	T	P	Q	Y	F	I	F	S	L	S
166	E	I	Q	N	G	S	D	V	Y	D	C	W	G	H	F
181	V	E	P	W	G	I	R	A	Y	I	T	W	I	T	V
196	G	I	F	L	L	P	V	I	I	L	M	I	C	Y	G
211	F	I	C	H	S	I	W	K	N	F	K	S	K	T	K
226	R	G	T	L	H	S	T	K	<b>D</b>	<b>R</b>	<b>M</b>	<b>I</b>	<b>G</b>	<b>K</b>	<b>G</b>
241	<b>S</b>	<b>V</b>	<b>S</b>	<b>S</b>	<b>V</b>	<b>T</b>	<b>I</b>	<b>I</b>	<b>S</b>	<b>R</b>	<b>A</b>	<b>K</b>	<b>L</b>	<b>R</b>	<b>T</b>
256	<b>V</b>	<b>K</b>	<b>M</b>	<b>T</b>	<b>F</b>	<b>V</b>	<b>I</b>	<b>V</b>	<b>L</b>	<b>A</b>	<b>Y</b>	<b>I</b>	<b>V</b>	<b>C</b>	<b>W</b>
271	<b>A</b>	<b>P</b>	<b>F</b>	<b>F</b>	<b>I</b>	<b>V</b>	<b>Q</b>	<b>M</b>	<b>W</b>	<b>S</b>	<b>V</b>	<b>W</b>	<b>D</b>	<b>E</b>	<b>N</b>
286	<b>F</b>	<b>S</b>	<b>W</b>	<b>D</b>	<b>D</b>	<b>S</b>	<b>E</b>	<b>N</b>	<b>A</b>	<b>A</b>	<b>V</b>	<b>T</b>	<b>L</b>	<b>S</b>	<b>A</b>
301	<b>L</b>	<b>L</b>	<b>A</b>	<b>S</b>	<b>L</b>	<b>N</b>	<b>S</b>	<b>C</b>	<b>C</b>	<b>N</b>	<b>P</b>	<b>W</b>	<b>I</b>	<b>Y</b>	<b>M</b>
316	<b>L</b>	<b>F</b>	<b>S</b>	<b>G</b>	<b>H</b>	<b>L</b>	<b>L</b>	<b>Y</b>	<b>D</b>	<b>F</b>	<b>L</b>	<b>A</b>	<b>C</b>	<b>F</b>	<b>P</b>
331	<b>C</b>	<b>W</b>	<b>N</b>	<b>K</b>	<b>P</b>	<b>R</b>	<b>N</b>	<b>T</b>	<b>L</b>	<b>H</b>	<b>K</b>	<b>V</b>	<b>D</b>	<b>S</b>	<b>D</b>
346	S	<b>S</b>	<b>I</b>	<b>R</b>	<b>R</b>	<b>N</b>	<b>T</b>	<b>L</b>	<b>L</b>	<b>S</b>	<b>K</b>	<b>L</b>	<b>A</b>	<b>T</b>	<b>V</b>
361	<b>R</b>	<b>T</b>	<b>K</b>	<b>D</b>	<b>G</b>	<b>F</b>	<b>D</b>	<b>S</b>	<b>W</b>	<b>K</b>	<b>D</b>	<b>P</b>	<b>C</b>	<b>N</b>	<b>S</b>
376	<b>R</b>	<b>K</b>	<b>S</b>	<b>S</b>	<b>Q</b>	<b>S</b>	<b>L</b>	<b>G</b>	<b>L</b>	<b>D</b>	<b>F</b>	<b>S</b>	<b>R</b>	<b>K</b>	<b>S</b>
391	<b>S</b>	<b>Q</b>	<b>C</b>	<b>L</b>	<b>Q</b>	<b>L</b>	<b>D</b>	<b>C</b>	*						

Figure 1. Amino acid sequence for the V1a-like receptor protein (VTR). Underlined portions signify transmembrane domains, and bold indicates where the canonical form extends past the truncated form. Colored portions are the regions targeted by the custom anti-VTR antibodies used in the current experiment: Blue specifies the epitope for the canonical-specific antibody, and green specifies the epitope for the dual antibody targeting both canonical and truncated forms of the receptor (see methods).



### ***Goldfish social context is determined by visual and olfactory cues***

Goldfish live in shoals and their social behavior is sensitive to both visual and olfactory cues, which are especially important during courtship (Kobayashi et al., 2002). When mating, male goldfish chase females in a scramble competition system that depends heavily on the social context determined by pheromone cues (Appelt and Sorensen, 2007). Before ovulating, female goldfish release the pheromone 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17,20-BP) into the water, which prompts male goldfish to produce more testosterone (Sorensen et al., 1989). Several hours later, during ovulation, female goldfish release another pheromone, prostaglandin F2 $\alpha$  (PGF2 $\alpha$ ), into the water, which triggers male courtship behaviors (Kobayashi et al., 2002; Sorensen et al., 1989). Appelt and Sorensen (2007) found that female goldfish released more PGF2 $\alpha$  when they were in a courtship context near males than when they were only near other females, especially when in proximity to spawning environments. This supports the important role of pheromones in goldfish courtship communication. Because pheromones give goldfish information about social context, and because VT systems have been shown to be sensitive to pheromones in other species like roughskin newts (Thompson and Moore, 2000; Thompson et al., 2008a), it is likely that the pheromone cues available to goldfish influence how vasotocin acts in the brain to affect social behavior.

### ***Behavioral effects of VT in the male goldfish***

As briefly mentioned above, recent work has investigated the role of VT in modulating goldfish social behavior. Approach and withdrawal behaviors are conserved processes that are essential for most social experiences (Thompson and Walton, 2009).

Thompson and Walton (2004) showed that in male goldfish, centrally-injected VT inhibited social approach towards other males, particularly during the breeding season, and an antagonist promoted social approach. This indicates that VT is associated with broad sociality and investigation, at least in a seasonal, male-male context (Thompson and Walton, 2004). Furthermore, Thompson et al. (2008b) characterized the goldfish neural circuits responsible for these inhibitory effects by centrally infusing VT into either forebrain or hindbrain regions and showing males a visual cue of another male. They demonstrated that hindbrain VT infusions inhibited male-male approach at a lower dose than did forebrain infusions, suggesting that VT works through the more conserved neural circuit in the hindbrain to inhibit approach to males. Mangiamele et al. (2013) also demonstrated through in situ hybridization that exposure to the male pheromone androstenedione (AD) yielded greater gene expression for VT neurons and, like VT, inhibited social approach, suggesting that AD may typically drive the VT circuit that promotes social withdrawal. Importantly, VT has been shown not to affect general motor or arousal activity, meaning that its effects on behavior are specific to social responses (Thompson and Walton, 2004).

Less is known about VT's modulation of goldfish behavior when it works through its neural circuit in the forebrain, though forebrain regions are closely related to mating and courtship behaviors in many vertebrates, including the butterflyfish, where VT immunoreactivity is associated with increased sociality and mating behaviors (Dewan et al., 2011). It is possible that when goldfish are in a courtship context, VT works through the forebrain circuit to promote approach to females. However, despite a large amount of evidence for context-dependent VT effects in other species, very little research has focused on whether goldfish respond differently to VT depending on the social context (Thompson

and Walton, 2004). Visual and olfactory social cues might alter VT circuitry, perhaps by mediating signaling pathways, receptor expression, and/or receptor constructs through dimerization of the canonical and truncated forms of the V1a-like receptor. In the current study, we hypothesized that VT might produce context-specific behavioral effects on goldfish through context-dependent modulation of V1a-like receptors.

### ***The current study***

The current study has three central goals: (1) To determine if both canonical and truncated V1a-like receptors (VTR) are translated in male goldfish, (2) To characterize the distributions of both VTR forms in the forebrain and hindbrain, and (3) To investigate the effects of VT on goldfish approach behaviors in social contexts related to courtship and reproduction. To address our first goal, we used western blotting with custom anti-VTR antibodies to quantify the sizes of these receptors in the goldfish brain. Given our expectation that both VTR genes are transcribed and translated into protein, we predicted that bands would be visible at the expected sizes for the canonical, truncated, and potentially dimerized forms of the receptor. To address our second goal, we used a standard immunohistochemistry protocol with the same custom anti-VTR antibodies to characterize VTR distribution throughout the brain. We expected that VTR immunoreactivity (VTR-ir) would exist throughout the hindbrain and the forebrain in regions associated with social behavior.

Finally, to address our third goal, we studied the effect of VT injection on male goldfish approach behaviors in a variety of social contexts. In Experiment 1, we predicted that peripherally-injected VT, like centrally-injected VT, would inhibit approach to other males in a dose-dependent manner. In Experiment 2, we predicted that VT might promote

social approach to females in a reproductive context, following pre-exposure to the female pre-ovulatory pheromone 17,20-BP. Finally, in Experiment 3, we predicted that pre-exposure to 17,20-BP would override VT's inhibitory effect on approach to males. Ultimately, we hypothesized that peripherally-injected VT would modulate goldfish social behavior in a context-specific manner, such that it would inhibit approach to males but might regulate approach behaviors differently in the presence of female visual and olfactory stimuli.

## **METHODS**

### ***Animal husbandry***

Adult goldfish, *Carassius auratus* (Blackwater Creek Fisheries, Eustis, FL), were housed in 200-gallon same-sex tanks at 20°C +/- 2°C, on a 14:10 hour light:dark schedule. Fish were fed goldfish pellets each day.

### ***Custom anti-VTR antibodies***

Two custom, anti-VTR polyclonal affinity purified primary antibodies were generated for this study (Lampire Biological Laboratories, Pipersville, PA). The “canonical” antibody, raised in guinea pig, is selective for a VTR epitope that only exists in the canonical form, while the “dual” antibody, raised in rabbit, recognizes an epitope that exists in both the truncated and canonical VTR forms (see Figure 1).

### ***VTR western blot***

Milting male goldfish were anesthetized in 0.1% ethyl 3-aminobenzoate methanesulfonate (MS-222) for 5 minutes after gill movement ceased. Brains were extracted

and either processed immediately or fresh-frozen at -80°C until use. Proteins were extracted from brain tissue following one of two protocols. In one extraction protocol, tissue was submerged in N-PER Neuronal Protein Extraction Reagent (Thermo Scientific, Rockford, IL) at a ratio of 1 g of tissue to 10 mL of reagent. Tissue was homogenized for 20 strokes, incubated on ice for 10 minutes, and centrifuged at 10,000  $\times$  g for 10 minutes at 4° C. Supernatant was collected as a “whole brain” tissue fraction. In the other extraction protocol, tissue was homogenized and cytosolic and plasma membrane fractions were separated using centrifugation protocols and solutions from a Plasma Membrane Protein Extraction Kit (Abcam, Cambridge, UK). A Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA) was used to determine the concentration of protein in each fraction. All protein fractions were stored at -20°C until use.

Protein fractions were denatured at 95°C for 5 minutes. Mini-Protein TGX Stain-Free Precast 12% gels (Bio-Rad, Hercules, CA) were loaded with protein fraction at concentrations ranging from 66 to 584 ng/ $\mu$ L in 25-30% loading buffer with dithiothreitol (Bio-Rad, Hercules, CA) in 0.3% Triton-X in PBS (PBST), alongside Precision Plus Protein Dual Color Standard #161-0394 (Bio-Rad, Hercules, CA). Gels were run on a Mini-Protein Tetra Cell (Bio-Rad, Hercules, CA) at 200V, 0.5 A, for 45 minutes. Next, proteins on gels were transferred to nitrocellulose membranes at 100 V, 0.35 A, for 1 hour, using a cold 25 mM Tris, 192 mM glycine, 20% v/v methanol SDS transfer buffer. Membranes were then incubated with a 5% nonfat milk block in 0.3% PBST for 1 hour at 50 RPM and 20°C, followed by overnight incubation with primary antibody in 0.3% PBST at 4°C. Primary antibodies included custom canonical and dual anti-VTR antibodies at concentrations ranging from 1:100 to 1:10,000. In an otherwise identical control protocol, primary antibodies were

preabsorbed with their immunizing antigens at a concentration of 100  $\mu$ M for 2 to 4 hours prior to incubation on membranes. Following primary antibody incubation, membranes underwent a series of washes with 0.3% PBST at 50 RPM and 20°C and were then incubated for 1 to 4 hours with secondary antibody at 1:4000 at 50 RMP and 20°C. Secondary antibodies included peroxidase-conjugated goat anti-rabbit antibody (Jackson Immuno Research, West Grove, PA) and peroxidase-conjugated goat anti-guinea pig antibody (EMD Millipore Corp, Billenca, MA). Membranes were then washed with 0.3% PBST at 50 RPM and 20°C and incubated in stable peroxide and luminol/enhancer solutions from a Supersignal West Pico PLUS Chemiluminescent Substrate Kit for 5 minutes (Thermo Scientific, Rockford, IL). A G:Box (Syngene, Cambridge, UK) and GeneSys software (Daly City, CA) were used to visualize protein on membranes at exposure times ranging from 6 seconds to 16 minutes.

### ***VTR immunohistochemistry***

Milting male goldfish were anesthetized in 0.1% MS-222 for 2 minutes and perfused with 4% paraformaldehyde (PFA). Brains were extracted, fixed in 4% PFA for 1 hour, and incubated overnight with a 30% sucrose solution at 4°C. Brains were then embedded in M-1 embedding medium (Thermo Scientific, Rockford, IL) and stored at -80°C until use. Brains were sectioned in 20  $\mu$ m coronal slices, thaw-mounted onto Tru-Bond subbed microscope slides (Electron Microscopy Sciences, Hatfield, PA), and stored at -80°C until processing.

Tissue was fixed with 4% PFA for 10 minutes, followed by a series of washes with PBS. Next, one of two protocols was followed. In one protocol, tissue was treated with 0.03% hydrogen peroxide for 2 minutes, washed with PBS, treated with 10 mM hot citric

acid 2 times for 5 minutes, and washed again with PBS. Tissue was then blocked with 10% donkey serum and PBS for 30 minutes and incubated overnight at 4°C with primary antibody in 0.3% PBST and 10% donkey serum. Primary antibodies included custom canonical and dual anti-VTR antibodies and anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (EMD Millipore Corp, Billerica, MA) at concentrations ranging from 1:500 to 1:1000. In an otherwise identical control protocol, primary antibodies were preabsorbed with their immunizing antigens at a concentration of 100  $\mu$ M for 2 to 4 hours prior to incubation on slides. Following primary antibody incubation, tissue was again washed with PBS and blocked for 15 minutes at 20°C with goat serum from a Tyramide Signal Amplification (TSA) kit (ThermoFisher Scientific, Waltham, MA). Tissue was then incubated for 1 hour at 20°C with secondary from the TSA kit, washed with PBS, treated with TSA kit tyramide reagents, washed with PBS, stained with DAPI, and stored at 4°C.

In the other protocol, tissue was blocked with 10% BSA in PBS for 2 hours following PFA fixation. It was then incubated overnight at 4°C with identical primary antibodies as in the first protocol at concentrations ranging from 1:200 to 1:1000. As in the first protocol, control primary antibodies were additionally preabsorbed with their immunizing antigens at a concentration of 100  $\mu$ M for 2 to 4 hours prior to incubation on slides. Following primary antibody incubation, tissue was washed with PBS, blocked with 5% goat serum in PBS for 30 minutes, and incubated for 1 hour in secondary antibody at 1:500. Secondary antibodies included goat anti-guinea pig antibody conjugated to Alexa Fluor 488, goat anti-rabbit antibody conjugated to Alexa Fluor 594, goat anti-mouse antibody conjugated to Alexa Fluor 488, and goat anti-mouse antibody conjugated to Alexa Fluor 594. Tissue was then washed with PBS, stained with DAPI, and stored at 4°C.

Slides were visualized on a fluorescent microscope. Images were taken at magnifications ranging from 4x to 40x using GRYPHAX microscope camera software (Jenoptik, Rochester Hills, MI). ImageJ (Bethesda, MD) was used to process figures and adjust for contrast.

### ***Behavioral testing***

#### **Experiment 1: Does peripherally-injected VT, like centrally-injected VT, inhibit approach to males in a dose-dependent manner?**

The night prior to testing, milt was confirmed in each focal male goldfish. Fish were then transferred to a 55-gallon tank with three milting conspecifics. Testing occurred the next morning between 9:00 a.m. and 1:00 p.m. As shown in Figure 2, focal fish were tested in 20-gallon tanks, separated on both sides from two 1.25-gallon stimulus fish compartments. Tank dividers were solid and clear, such that focal and stimulus fish could interact visually but not physically or via olfactory cues. Video-tracking Limelight software (Actimetrics Software, Wilmette, IL) was used to measure the amount of time the focal fish spent in a predetermined 4.5x8-inch interaction zone in closest proximity to the stimulus fish compartment. In addition, the number of crossings into this zone was recorded as a measure of general activity.



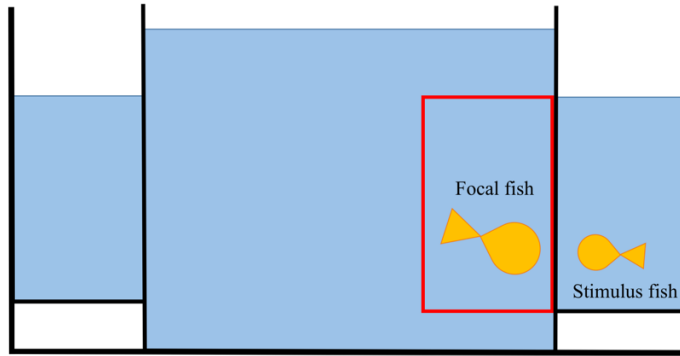


Figure 2. Social approach behavioral paradigm tanks. Focal fish were placed in the central 20-gallon chamber, and stimulus fish were placed in the 1.25-gallon compartment on the side of the tank least preferred by the focal fish during baseline. Right interaction zone (4.5x8 inches) is outlined in red.

Figure 3 gives the general timeline for behavioral testing. To account for individual variation and control for order effects, each fish was tested twice and served as its own control. The focal fish was placed in the testing tank for 15 minutes of habituation, followed by 20 minutes of baseline testing. Time spent in each interaction zone was examined to determine initial side preference. Time spent and number of crossings into the least preferred interaction zone were recorded as T1 baseline. The focal fish was then given a 100  $\mu$ l intraperitoneal injection of saline vehicle. Following 5 minutes of recovery, a non-milting male stimulus fish was introduced to the compartment on the side least preferred by the focal fish during baseline. Time and crossings in the interaction zone closest to the stimulus fish were recorded for 20 minutes (T1 testing) before the stimulus fish was removed and the focal fish re-habituated to the empty tank for 15 minutes. The procedure was then repeated, comprising 20 minutes of baseline testing (T2 baseline), a 100  $\mu$ l intraperitoneal injection of either saline control (veh/veh condition) or 1 or 5  $\mu$ g/gbw VT (Sigma-Aldrich, Darmstadt, Germany) (veh/VT condition), 5 minutes of recovery, the re-introduction of the stimulus male to the least preferred side, and 20 minutes of social approach testing (T2 testing). When VT was injected at a dose of 1  $\mu$ g/gbw, n=8 for both veh/veh and veh/VT groups. When VT

was injected at a dose of 5 µg/gbw, n=6 for both veh/veh and veh/VT groups. Based on behavioral responses in Experiment 1, only the higher dose of 5 µg/gbw VT was used in all future behavioral experiments.

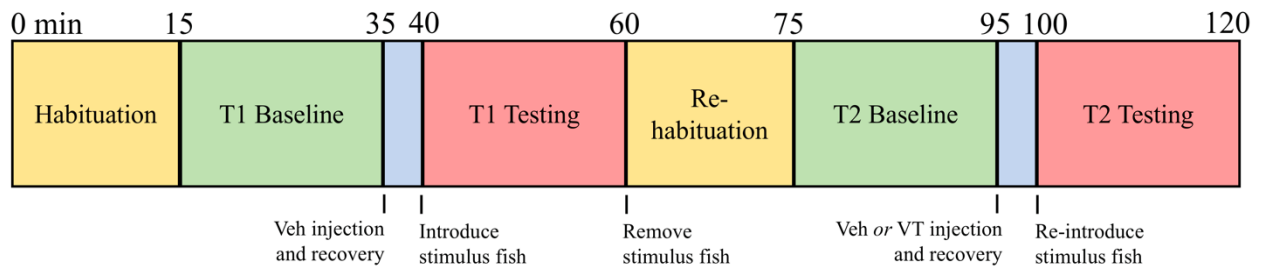


Figure 3. General timeline for behavioral testing: 15 minutes of habituation, 20 minutes of T1 baseline, intraperitoneal injection of saline vehicle, 5 minutes of recovery, introduction of stimulus male to least preferred side, 20 minutes of T1 testing, removal of stimulus male, 15 minutes of re-habitation, 20 minutes of T2 baseline, intraperitoneal injection of either saline vehicle or VT, 5 minutes of recovery, re-introduction of stimulus male to least preferred side, and 20 minutes of T2 testing.

## Experiment 2: Does VT promote approach to females in a reproductive context, as constructed by olfactory and visual cues?

The night prior to testing, milt was confirmed in each focal male goldfish. Goldfish were then transferred overnight to a bucket with  $5 \times 10^{-9}$  M 17,20-BP (Steraloids Inc., Newport, RI) in 4 L of water, alongside one milting conspecific. Testing occurred the next morning between 9 a.m. and 1 p.m. following a nearly identical protocol to Experiment 1 (see Figure 3) with two key differences. First, stimulus fish in Experiment 2 were adult female goldfish injected with 5 µl of 5 mg/ml lutalyse (Zoetis, Parsippany-Troy Hills, NJ) 30-40 minutes before T1 testing to ensure ovulation. Second, the dividers between the central chamber and the stimulus fish compartments were clear and perforated, such that fish could interact both visually and via pheromone cues. In total, n=6 fish for both veh/veh and veh/VT groups.

### **Experiment 3: Does pre-exposure to 17,20-BP override VT's inhibitory effect on approach to males?**

In Experiment 3a, milt was measured in each focal male goldfish the night prior to testing (Day 1 milt). Fish were then transferred and isolated overnight in a bucket with either  $5 \times 10^{-9}$  M 17,20-BP or the same concentration of ethanol vehicle in 4 L of water. Testing occurred the next morning between 9 a.m. and 1 p.m. following an identical protocol to Experiment 1 (see Figure 3). Day 2 milt was measured immediately following T2 testing. In total, n=9 fish for the ethanol exposed, vehicle injected (eth/veh/veh) and ethanol exposed, VT injected (eth/veh/VT) groups, and n=8 fish for the pheromone exposed, vehicle injected (17,20-BP/veh/veh) and pheromone exposed, VT injected (17,20-BP/veh/VT) groups.

Due to the stress associated with overnight isolation and multiple injections during Experiment 3a, Experiment 3b followed an alternative paradigm designed to reduce stress levels and increase response to 17,20-BP. Preliminary evidence from Love Avril '22 demonstrated no significant change in approach responses from T1 to T2 in a similar procedure to Experiment 1 in which fish were only injected once with vehicle prior to T2 testing. As in Experiment 3a, milt was confirmed and measured in each focal male goldfish the night prior to testing (Day 1 milt). Fish were then transferred overnight to a bucket with a higher concentration of  $10^{-8}$  M 17,20-BP or the same concentration of ethanol vehicle in 4 L of water, alongside one milting conspecific. Testing occurred 14 hours later between 8 a.m. and 12 p.m. following a similar protocol to Experiment 1 (see Figure 3), and Day 2 milt was measured immediately following T2 testing. Importantly, all focal fish in these groups were only given one intraperitoneal injection of either either saline vehicle or 5 µg/gbw VT

following T2 baseline; no injections occurred following T1 baseline in this experiment. In total, n=7 fish across all four groups (eth/veh, eth/VT, 17,20-BP/veh, 17,20-BP/VT).

### ***Behavioral data analysis***

Data were analyzed using SPSS Statistics (IBM Corporation; Armonk, NY), and figures were generated using GraphPad Prism 7 (Graphpad; La Jolla, CA). Time and crossings during T1 and T2 baseline were subtracted from these values during T1 and T2 testing to create corrected scores for each fish. A total of 1 fish was excluded (from Experiment 2) because it did not move during testing, indicating a severe stress response. We initially inspected time, crossings, and milt data for outliers, normality, and equality of variance and found that several variables across experiments did not meet the necessary assumptions for parametric statistical analysis. Thus, to facilitate non-parametric analysis, we subtracted T1 corrected scores from T2 corrected scores to represent change following VT injection. Specific T1 and T2 scores are shown in Supplementary Figures S1-S3. For those experiments where milt was measured, difference scores were calculated by subtracting Day 1 milt scores from Day 2 milt scores. Difference scores for time in zone, crossings, and milt were compared across groups using Kruskal–Wallis H tests. For all hypothesis tests, we set alpha at 0.05.

## **RESULTS**

### ***Western blot analysis of canonical and truncated VTR***

Western blot data for the dual antibody revealed protein bands near 30 kD, 40 kD, between 50 kD and 60 kD, and 150 kD (see Figure 4a). Preabsorption with the immunizing antigen dramatically reduced signal for the bands at 30 kD, between 50 kD and 60 kD, and

150 kD. For the canonical antibody, protein bands were observed near 30 kD, 40 kD, 45 kD, between 50 kD and 60 kD, and 75 kD (see Figure 4b). Preabsorption with the immunizing antigen dramatically reduced signal for all bands.

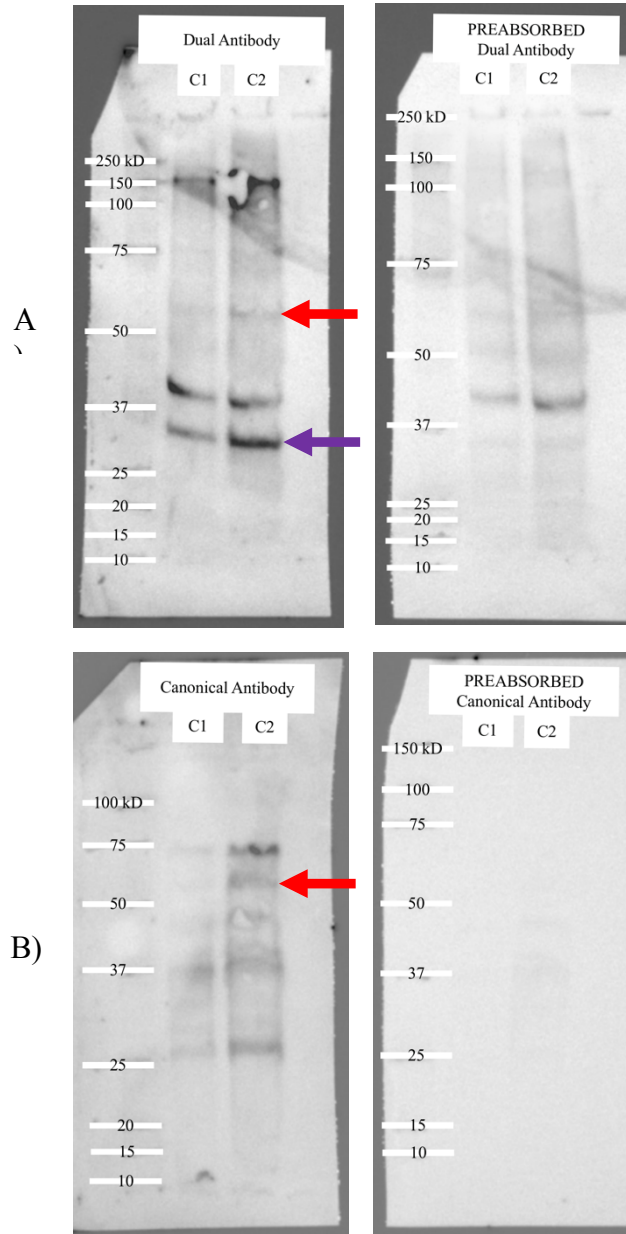


Figure 4. Western blot data for the dual (A) and canonical (B) antibodies at 1:1000 using a whole-brain protein fraction at low (C1) and high (C2) protein concentrations extracted from a milting male with N-PER reagent. Red and purple arrows mark bands near the expected sizes for the canonical and truncated VTR respectively.

## *VTR immunohistochemistry*

### **Verification of antibody specificity**

Preabsorption with VT immunizing antigens dramatically reduced VTR-ir for both dual and canonical antibodies (see Figure 5).

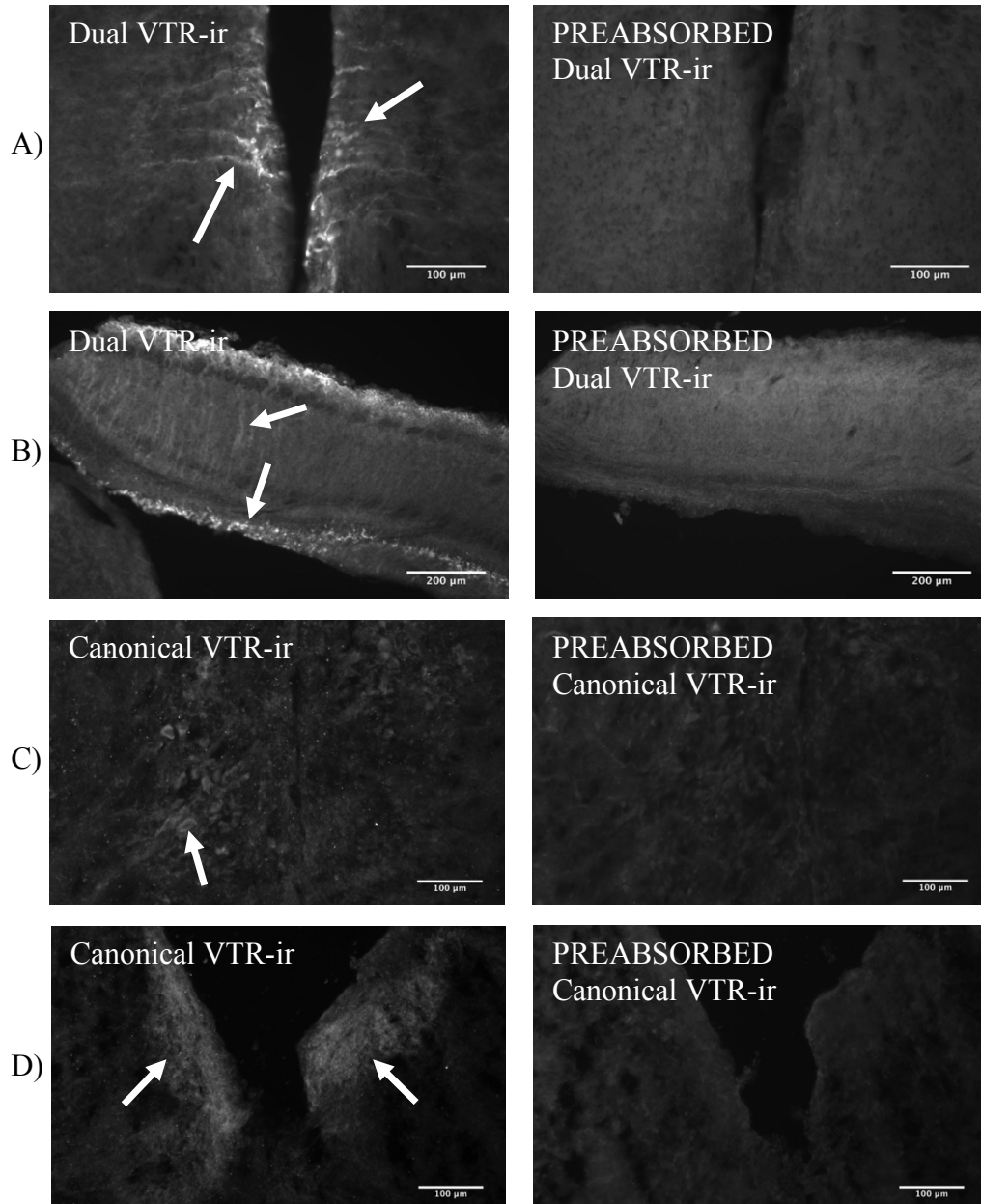


Figure 5. VTR-ir at the midline (A), optic tectum (B), preoptic area (POA) (C), and area postrema (D) for dual (1:500; TSA protocol) and canonical (1:200; non-TSA protocol) antibodies, following identical procedures with or without immunizing antigen preabsorption. Arrows indicate increased VTR-ir in the non-preabsorbed condition. Images taken at 20x (A, C, D) and 10x (B).

### **Characterization of canonical and truncated VTR**

VTR-ir was observed throughout the forebrain and hindbrain, particularly in the telencephalon, preoptic area (POA), midline, optic tectum, cerebellum, dorsal motor vagus, and area postrema (see Figure 6). VTR-ir from the dual and canonical antibodies revealed overlapping patterns, with stronger signal generally visible in fibers for the dual antibody and in cell bodies for the canonical antibody.

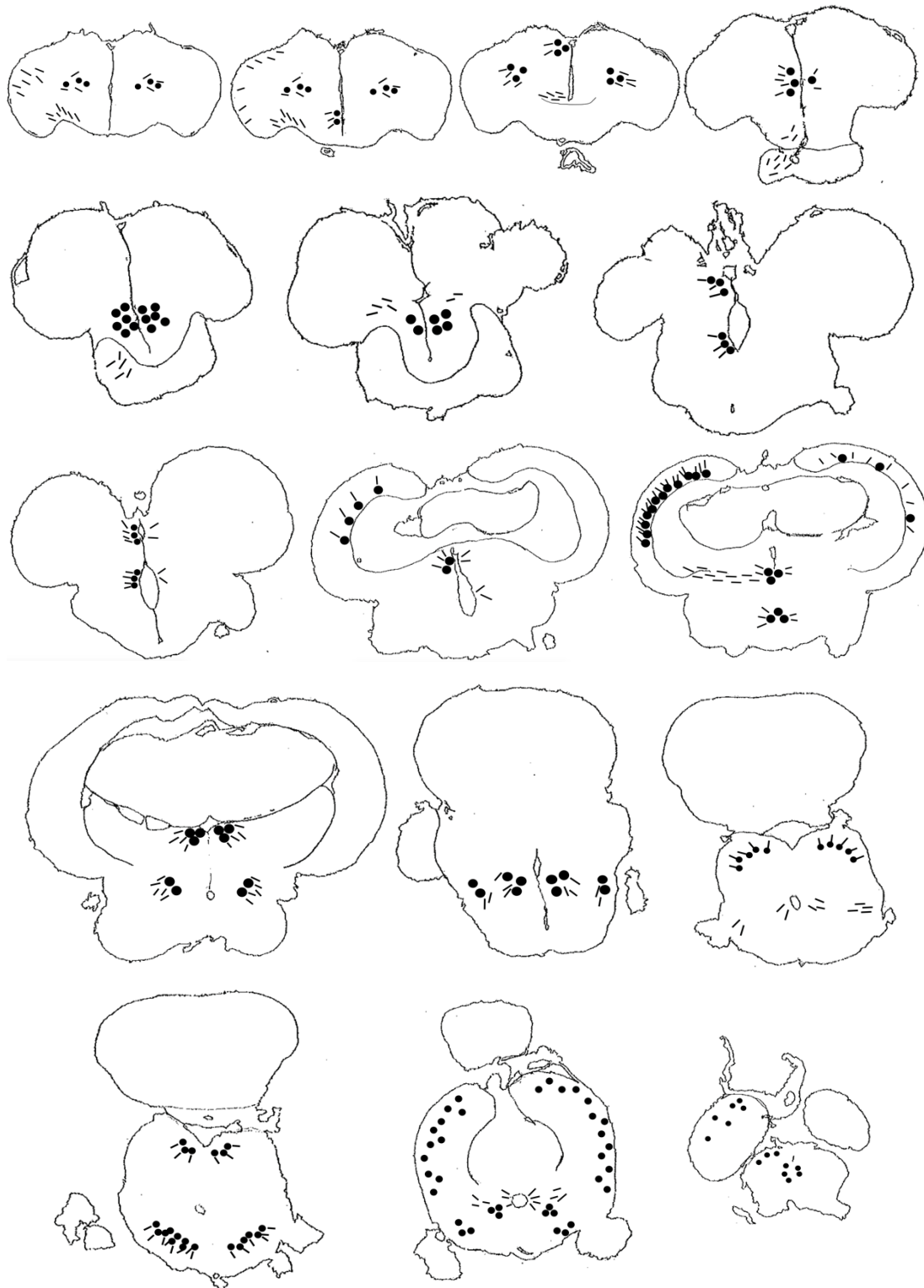


Figure 6. VTR expression throughout the brains of milting males (n=2) showing VTR-ir from the dual (left of midline) and canonical (right of midline) antibodies. Lines indicate fiber projections, and circles indicate cell bodies. Dual and canonical antibody signal overlapped but showed different patterns of intensity.



### *Forebrain through POA*

VTR-ir was visible in fibrous extensions throughout telencephalon, particularly in central, dorsal, and ventral regions. Fibers in the central telencephalon colocalized for the two antibodies (see Figure 7a), but were stronger and more clearly defined laterally and along the midline for the dual antibody. The dual antibody revealed particularly strong extensions from the midline in the supra commissural nucleus of the ventral telencephalon (VS) (see Figure 7b). Strong VTR-ir was observed in fibers extending from the midline throughout the forebrain and midbrain, particularly for the dual antibody (see Figure 7c). VTR-ir was also observed in fibers and cell bodies in the POA. Signal from magnocellular and gigantocellular POA neurons colocalized between antibodies, with stronger intensity for the canonical antibody along cell membranes (see Figure 7d).

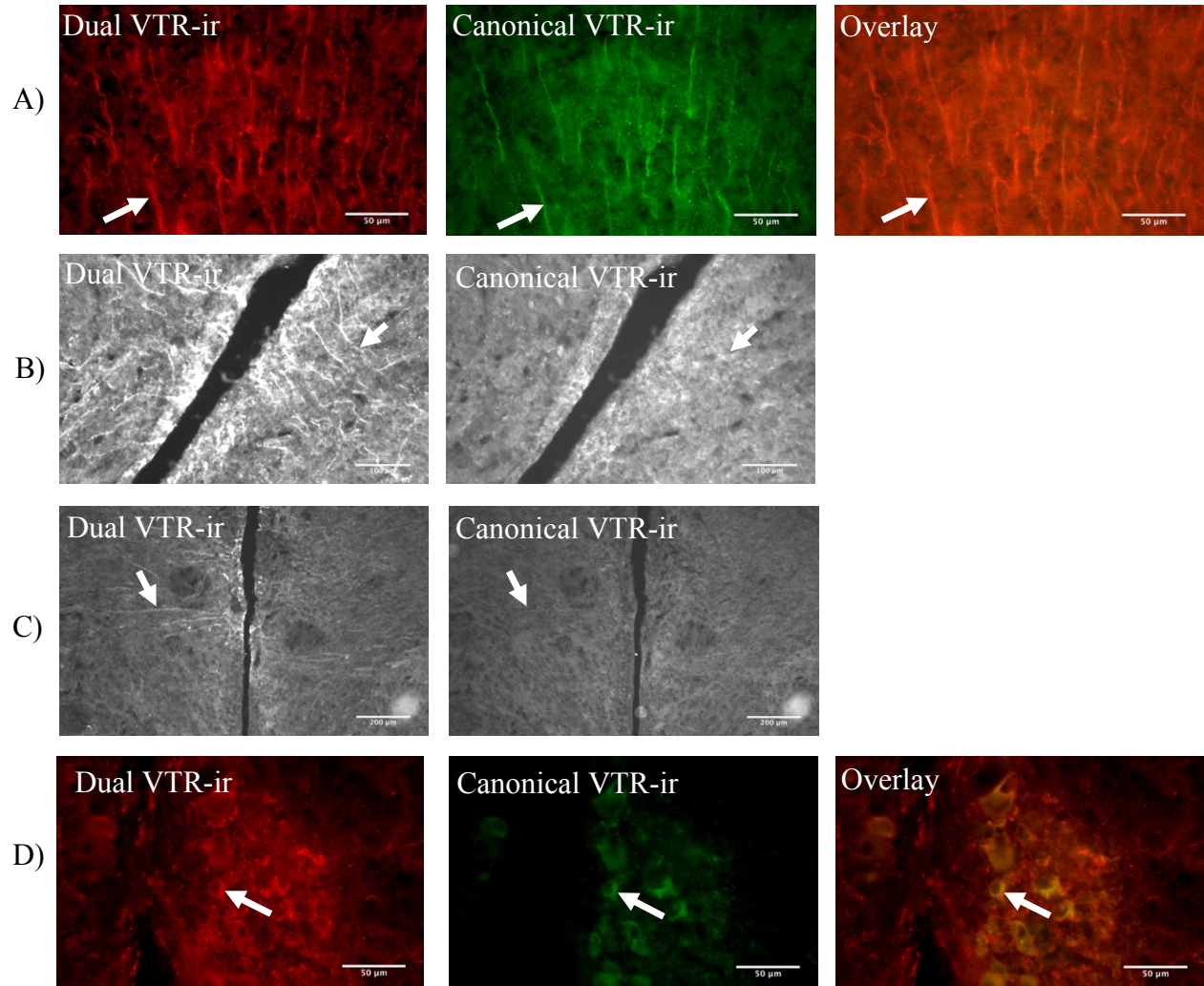


Figure 7. VTR-ir in the central telencephalon (A), VS (B), midline (C), and POA (D) of a milting male using the non-TSA protocol for the dual and canonical antibodies at 1:200. Arrows indicate colocalized VTR-ir. Images taken at 40x (A, D), 20x (B), and 10x (C).

### *Optic tectum and cerebellum*

VTR-ir was visible in cells and fibers throughout the optic tectum, particularly for the dual antibody (see Figure 8a). Additionally, VTR-ir was seen in cell membranes and fibers in the cerebellum for the canonical antibody (see Figure 8b).

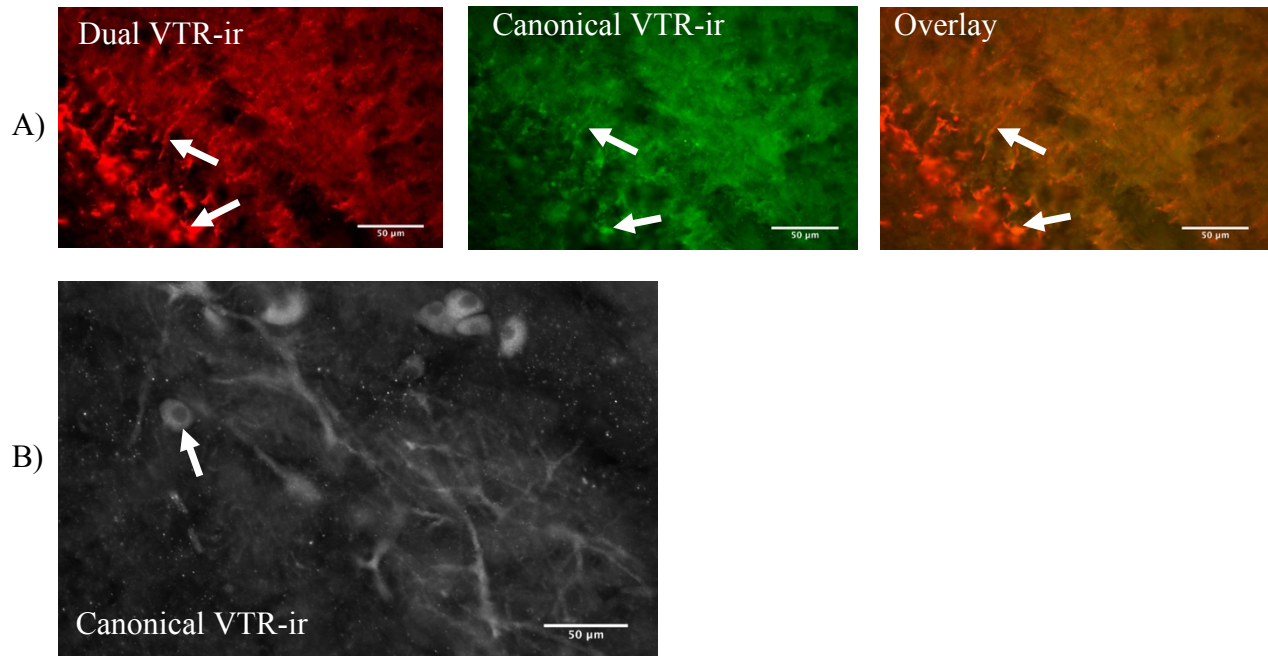


Figure 8. VTR-ir in the optic tectum (A) and cerebellum (B) of a milting male using the non-TSA protocol for the dual and canonical antibodies at 1:200. Arrows indicate colocalized VTR-ir in (A) and VTR-ir along a cell body in (B). Images taken at 40x.

### *Hindbrain*

VTR-ir was observed in cell bodies and neuropil in the dorsal motor vagus for both dual and canonical antibodies but was more intense for the canonical antibody (see Figure 9a). Additionally, colocalized VTR-ir was observed in cell bodies and projections in the area postrema, with stronger signal observed for the dual antibody (see Figure 9b).

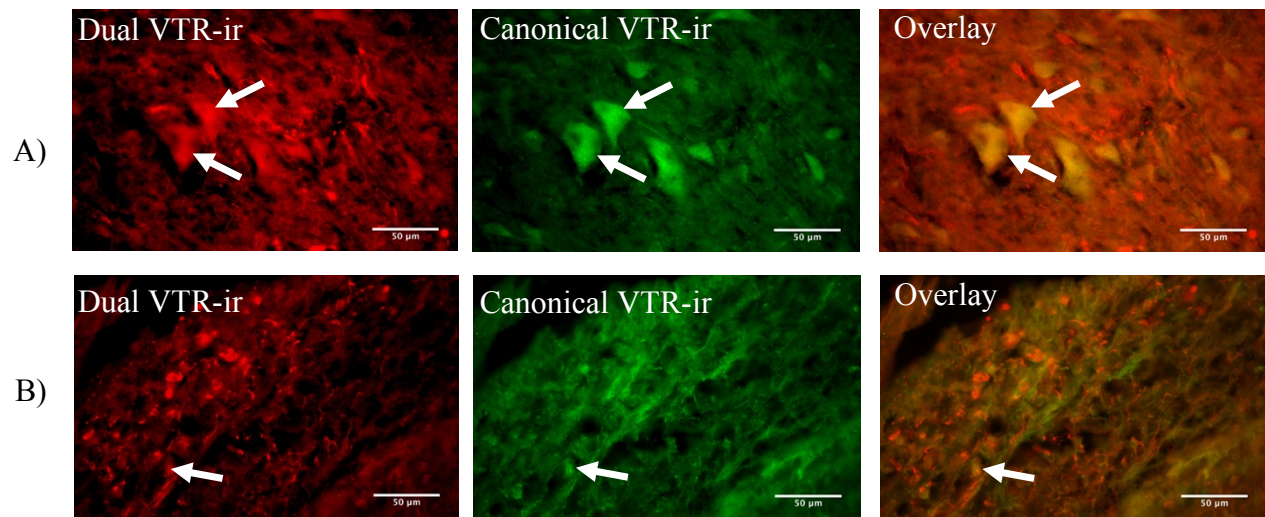


Figure 9. VTR-ir in the dorsal motor vagus (A) and area postrema (B) of a milting male using the non-TSA protocol for the dual and canonical antibodies at 1:200. Arrows indicate colocalized VTR-ir. Images taken at 40x.

### Colocalization of VTR and radial glial cell signal

Canonical VTR-ir colocalized with an anti-GFAP antibody, a marker for radial glial cells, in the telencephalon (see Figure 10).

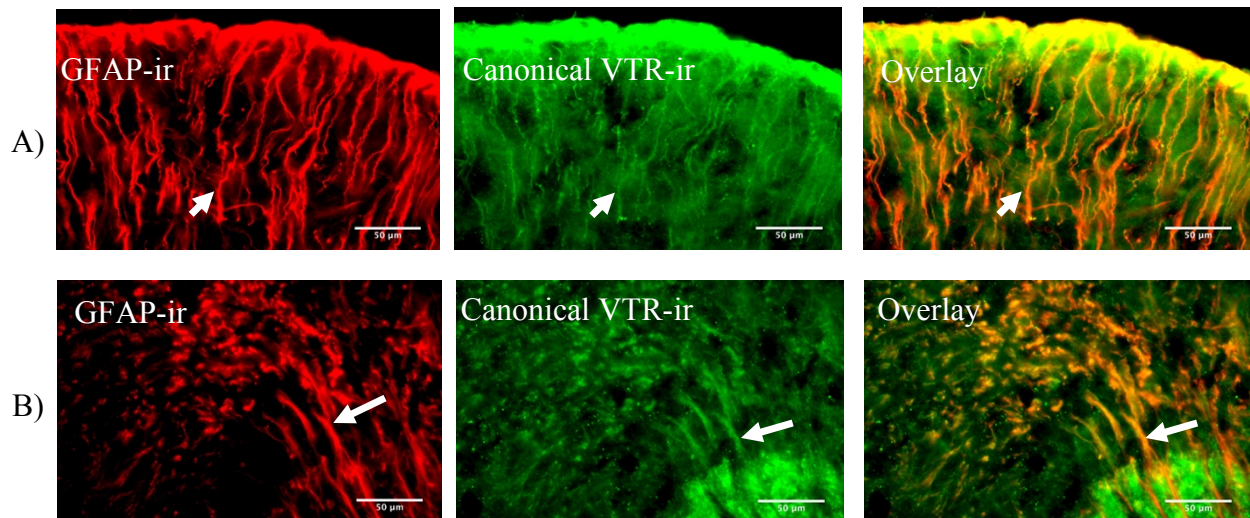


Figure 10. VTR-ir in the dorsolateral telencephalon (A) and anterior telencephalon (B) of a milting male using the non-TSA protocol for the GFAP (1:500) and canonical (1:200) antibodies. Arrows indicate colocalized VTR-ir and GFAP-ir. Images taken at 40x.

## Behavioral testing

### Experiment 1

As predicted, VT at 1  $\mu\text{g}/\text{gbw}$  tended to reduce time near the stimulus fish on T2 (see Figure 11), though this difference was not statistically significant; a Kruskal–Wallis H test comparing T2-T1 differences revealed no significant changes between groups for either time in zone or number of crossings ( $H = .121$ ,  $p = .728$ ;  $H = 3.429$ ,  $p = .064$ ). At 5  $\mu\text{g}/\text{gbw}$ , VT significantly reduced time spent near the stimulus fish on T2 (see Figure 11); a Kruskal–Wallis H test comparing T2-T1 differences for time in zone revealed significantly higher differences in the veh/VT group ( $H = 5.769$ ,  $p = .016$ ). A Kruskal–Wallis H test comparing T2-T1 differences in number of crossings revealed no significant differences between groups at 5  $\mu\text{g}/\text{gbw}$  VT ( $H = 2.077$ ,  $p = .150$ ).

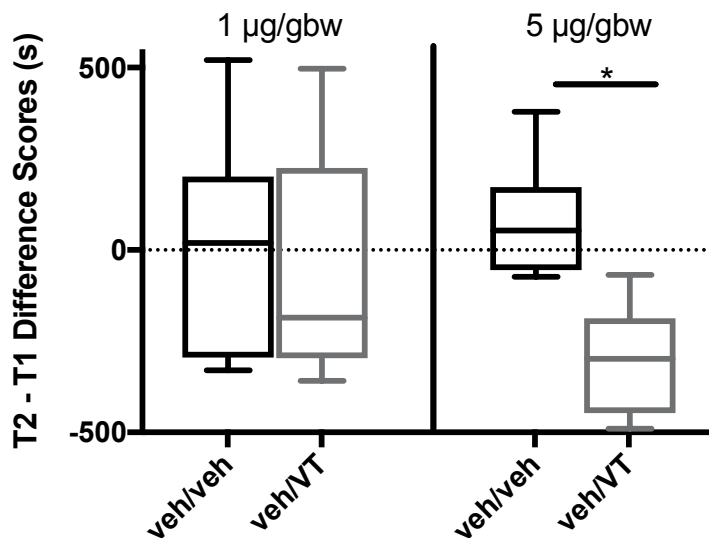


Figure 11. Approach to males at low and high VT doses. VT did not significantly affect behavior at the low dose, but T2-T1 differences were significantly larger for veh/VT fish at the high dose ( $H = 5.769$ ,  $p = .016$ ).

### Experiment 2

A Kruskal–Wallis H test comparing T2-T1 differences revealed no significant differences between groups for either time in zone or number of crossings ( $H = .000$ ,  $p =$



1.000;  $H = .923$ ,  $p = .337$ ; see Figure 12). Overall, male fish spent less time in the interaction zone near females than they did near males in Experiment 1 (see Supplementary Figures S1 and S2).

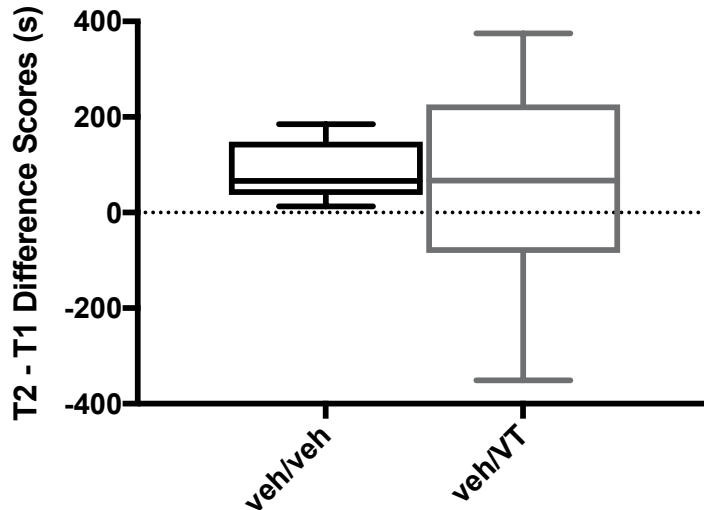


Figure 12. Approach to females following exposure to 17,20-BP. VT did not significantly affect behavior, but approach was low to begin with.

### Experiment 3a

There were no significant differences between groups for either time in zone or number of crossings ( $H = 5.13$ ,  $p = .163$ ;  $H = 1.521$ ,  $p = .678$ ; see Figure 13). Additionally, there were no significant differences between groups in expressible milt ( $H = 6.921$ ,  $p = .074$ ; see Figure 14).

### Experiment 3b

There were no significant differences between groups for time in zone ( $H = 2.949$ ,  $p = .400$ ; see Figure 13). Additionally, there were no significant differences between groups in expressible milt ( $H = 2.096$ ,  $p = .553$ ; see Figure 14).

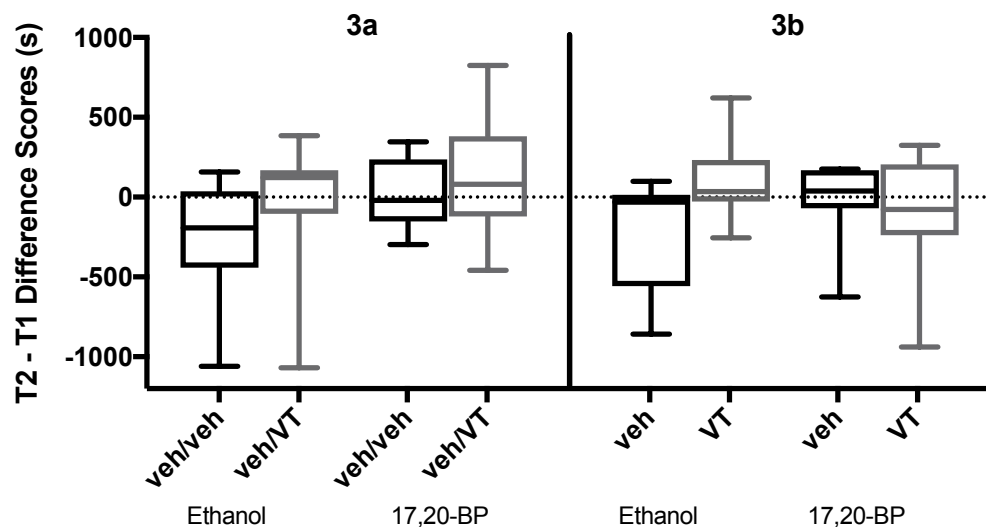


Figure 13. Approach to males following exposure to either 17,20-BP or an ethanol control. Neither VT nor pheromone exposure significantly affected behavior in either protocol.

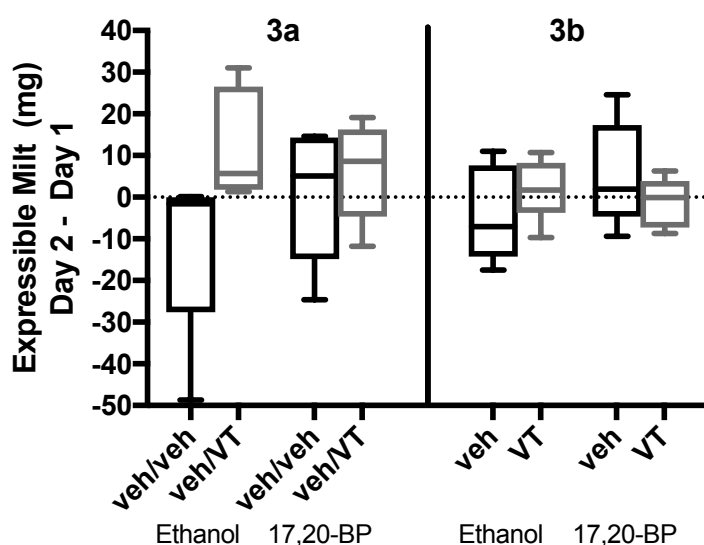


Figure 14. Milt differences between days in Experiment 3. There were no significant differences between groups for either protocol.

## DISCUSSION

The current study investigated context-dependent VT functioning in male goldfish by (1) determining if both canonical and truncated VTR are translated in the brain, (2) characterizing VTR distributions, and (3) examining whether VT's effects on social behaviors depend on social context. Western blot analysis indicated that both the canonical

and truncated VTR genes are likely translated into protein in the goldfish brain, though the presence of additional bands also suggests the possibility of non-specific binding.

Additionally, VTR-ir from the dual and canonical antibodies localized in overlapping distributions with differentiated patterns of signal intensity. The fact that both antibodies showed signal in the same regions suggests that this signal is VTR-related, as it is unlikely that both antibodies would recognize non-specific regions on unrelated proteins. Behavioral results indicated that VT inhibited approach to other males, but its effects on approach to females and in reproductive contexts requires further study, likely because protocols necessary to manipulate context and VT levels led to stress that altered baseline behavior.

***Both canonical and truncated VTR are likely expressed in the goldfish brain***

Recent unpublished work in the Thompson lab has sequenced two genes for VTR, one coding for a full-length canonical receptor and one for a truncated receptor that is unlikely to have ligand-binding capacity. However, the current study is among the first to examine whether both genes are actually translated into protein. We hypothesized that our western blot findings would support initial evidence from Andersen (2017) showing that both canonical and truncated receptors may exist in the goldfish brain. Thus, we expected to see bands near 45.44 kD and 26.69 kD, the predicted sizes for the canonical and truncated receptors respectively.

Western blot analysis for the dual antibody revealed bands near 30 kD, 40 kD, between 50 kD and 60 kD, and 150 kD. Signal was reduced in the preabsorbed condition for the bands near 30 kD, between 50 kD and 60 kD, and 150 kD, suggesting antibody specificity for VTR in all bands with the exception of 40 kD. The dual antibody recognizes



an epitope that exists in both VTR constructs. Thus, the presence of bands close to the predicted sizes for both canonical and truncated proteins provides compelling evidence that both forms of VTR are indeed translated in the goldfish brain. It is likely that the bands near 30 kD and between 50 kD and 60 kD correspond to the truncated and canonical forms of the receptor respectively. Additionally, the band at 150 kD may represent a dimerized form of the two proteins. This possibility for dimerization is supported by work from Zhu and Wess (1998), who showed that a truncated version of the V2 receptor can dimerize with the canonical V2 receptor. Furthermore, the Thompson lab also has preliminary evidence for a 5' truncated V1a-related gene, though its full length has not yet been determined. If translated, it could give rise to one of the additional bands observed.

Western blot analysis for the canonical antibody indicated bands near 30 kD, 40 kD, 45 kD, between 50 kD and 60 kD, and 75 kD. Preabsorption dramatically reduced signal for all bands, suggesting that the signal was specific to VTR. The canonical antibody recognized an epitope specific to the full-length VTR construct. Due to the differences in scale between western blots for the dual and canonical antibodies, it is difficult to determine if the bands visible between 50 kD and 60 kD for both antibodies are picking up a protein at the same size. Presuming that they are, it is likely that both of these bands correspond to the canonical form of the receptor. Furthermore, the band at 75 kD for the canonical antibody may represent additional dimerized VTR forms, while the smaller bands may indicate protein degradation. However, the presence of these additional bands may also suggest non-specific binding. Thus, future work should continue to fine-tune western blot protocols with both antibodies to accurately characterize the size of VTR constructs throughout the brain, as well as control for antibody specificity. Together, the current western blot results align with

similar evidence from Andersen (2017) and support our hypothesis that both canonical and truncated VTR genes are likely translated into protein in the goldfish brain.

***Canonical and truncated VTR localize in overlapping distributions with differentiated patterns of signal intensity***

If canonical, truncated, and dimerized forms of the VTR protein exist in the brain, it is important to examine why translating a non-functional form of VTR may contribute to the specificity of VT's behavioral effects. Zhu and Wess (1998) showed that the dimerization of truncated and canonical V2 receptors decreased sensitivity to VP. It is therefore possible that a similar process could occur for V1a-like receptors in goldfish brains. Specifically, differing amounts of truncated receptors in different brain regions may alter how much of the canonical receptor is trafficked to the membrane, mediating VT binding and producing diverse behavioral effects. Such a process may be regulated by many factors, including social context. However, before examining the possibility for such complicated context-dependent receptor modulation, we must first characterize VTR distributions throughout the brain. We did so using immunohistochemistry with custom anti-VTR dual and canonical antibodies. Signal observed using the dual antibody may indicate the presence of both truncated and canonical VTR proteins, as it recognizes an epitope region common to both, while signal for the canonical antibody would be specific to the full-length, fully-functioning canonical form of VTR.

Control results demonstrated that VTR-ir was dramatically reduced in the preabsorbed condition, verifying that both antibodies were specific to the VTR epitope used to generate them. As hypothesized, VTR-ir was observed in numerous brain areas involved

in processing social stimuli throughout both the hindbrain and the forebrain, including several nodes of the SBN. These findings are consistent with widespread evidence that VT affects social behavior (Albers, 2015). Overlapping signal was observed in fibers in the central, dorsal, and ventral telencephalon and along the midline for both canonical and dual antibodies, and the dual antibody showed particularly strong midline extensions in the VS. Colocalized signal was also observed in the POA, the node of the SBN where VT is exclusively produced in teleosts (Thompson and Walton, 2009). Signal surrounding the cell bodies of gigantocellular preoptic neurons was seen for both antibodies but appeared stronger for the canonical antibody. The presence of signal surrounding cell bodies in the POA is exciting, as VTR is a membrane-bound G-protein coupled receptor. In the optic tectum, an area associated with visual processing of social stimuli such as other fish, colocalized signal was visible in cell bodies and fibers, appearing particularly intense for the dual antibody. Overlapping signal was also observed throughout hindbrain circuits in areas characterized by Thompson and Walton (2004), including in the dorsal motor vagus, where signal was particularly strong in cell bodies for the canonical antibody. In the area postrema, signal was also colocalized and appeared stronger in cell bodies and fibers for the dual antibody.

Overall, immunohistochemistry results revealed overlapping localization for VTR constructs targeted by the dual and canonical antibodies. The colocalization of signal is expected because the dual antibody should recognize both constructs and therefore appear alongside the canonical antibody. The overlapping nature of the distribution patterns adds further support that these two custom antibodies indeed target the VTR protein, as it is unlikely that two distant epitope regions of the same antibody would recognize a similar, unrelated protein.

The VTR distributions observed in the current study are consistent with findings from Kline et al. (2011), who performed the first complete VTR characterization in teleosts. Using a custom antibody in the brains of rock hind, Kline et al. (2011) saw strong VTR-ir throughout the forebrain and the hindbrain, including in the olfactory bulbs, dorsal and ventral telencephalon, preoptic nucleus, optic tectum, cerebellum, and posterior regions. Kline et al. (2011) also observed VTR expression in additional areas associated with behavior, olfaction, and reproduction, including the nucleus corticalis, torus semicircularis, commissural preglomerular nucleus, granular eminence, and pituitary. These regions should be of particular interest throughout future work characterizing VTR-ir in the brains of goldfish and other teleosts.

Furthermore, it is important to note that the two antibodies in the current study demonstrated differentiated patterns of signal intensity, with the canonical antibody generally showing stronger signal in cell bodies in the POA and hindbrain and the dual antibody showing stronger signal along fibers. The fact that the canonical antibody appears stronger in cell bodies suggests that it is not simply the case that the dual antibody is a better antibody, but that these antibodies in fact identify unique patterns of VTR-ir in the brain. Areas where the dual antibody is stronger than the canonical antibody suggest that truncated receptors are more dominant than canonical receptors in these regions. In regions with more truncated receptors, they may dimerize with the functional canonical receptor, impairing VT's ability to bind and therefore inhibiting VT effects on functions mediated by actions in those areas. Because VT's behavioral effects depend on the pattern of neural activation throughout the SBN (Goodson and Kabelik, 2009), such differences in VT actions in specific areas may ultimately alter VT's effects on social behavior. It is thus possible that while VT has been

shown to inhibit social approach towards other males when acting in a hindbrain neural circuit (Thompson et al., 2008b), it may promote other effects if that circuit is inhibited and others disinhibited in particular contexts. This may be particularly true in reproductive contexts in which VT has been associated with the promotion of courtship in the forebrain of other teleosts (Dewan et al., 2011).

VTR-ir in fibers additionally colocalized with GFAP-ir, a marker for radial glial cells, in several locations throughout the brain, particularly for the canonical antibody in the telencephalon. This suggests that VTR may be found in radial glial cells in goldfish, complementing work by Nagarajan et al. (2016) demonstrating VTR-ir in glial cells in chickens. In teleosts, radial glial cells produce aromatase, the rate-limiting enzyme in the estrogen production pathway (Forlano et al., 2001). The colocalization of VTR in radial glial cells is thus consistent with widespread evidence that VT functioning is related to sex- and seasonal-specificity (Goodson and Bass, 2001). This colocalization between VTR-ir and GFAP-ir may indicate an interaction between neuropeptide systems and sex steroid systems, which work together to produce diversity in social behaviors. Additionally, it is important to note that VT's functions are diverse and not isolated to those related to social behavior; the VP/VT neuropeptide family has been implicated in behavioral and physiological functions as widespread as the cardiovascular system (Feuerstein et al., 1984), sleep (Goldstein, 1983), appetite regulation (Nagarajan et al., 2016), memory, and antidiuretic functioning (reviewed in Wied et al., 1984). Surprisingly, less colocalization was observed between the dual antibody and radial glial cells in the current study, despite seeing stronger VTR-ir in fibers for the dual compared to canonical antibody. However, we also did not observe GFAP-ir in

some areas that we would expect in fish; thus, more characterization is needed to clarify the relationship between VTR and radial glial cells.

The observed localization patterns for canonical and truncated VTR in the current study are overlapping and exist in regions of the brain that influence social behaviors. Future work should continue to characterize VTR, specifically examining whether exposure to different visual and olfactory social stimuli modulates one or both receptor types throughout the brain. Such an investigation will complement the current study's results, providing greater insight into the mechanism through which context may mediate behavioral responsiveness to VT through modulation of its receptors.

***VT inhibits approach to males, but its effect on response to females and in reproductive contexts is still undetermined***

We finally explored whether social context alters VT's effects on sociability, with the expectation that if so, it may do so by modulating the expression of one or both forms of VTR in circuits that promote different behaviors. We found that peripherally-injected VT, in a dose-dependent fashion, inhibited approach to other males, consistent with findings by Thompson and Walton (2004) for central injections. Notably, a higher dose of VT was required to reach significance for peripheral than central injections, likely because the VT must cross the blood brain barrier in this case. Our work is now the first in goldfish to show that peripheral and central VT administration produce the same behavioral effects.

However, our attempts to determine if VT produces different effects towards females or towards other males after pre-ovulatory pheromone exposure were inconclusive, likely due to a stress response overriding any behavioral responsiveness to VT during testing. VT did

not appear to stimulate courtship-related approach to females, but approach to females was low to begin with. Furthermore, following pheromone exposure, neither VT injection nor pheromone exposure significantly affected approach to males. Importantly, 17,20-BP did not increase milt production as would be expected (Sorensen et al., 1989), implying that fish were not physiologically responsive to the pheromone exposure. Our initial experiments testing VT's effects towards males were the only behavioral tests in the current study that did not involve overnight pheromone exposure; thus, it is likely that the stress of being in buckets either in pairs or in isolation during pheromone exposure prevented fish from responding to either pheromone or VT as expected during behavioral testing. Even in the male-male test designed to be a less stressful paradigm with fewer injections, results from the initial male-male experiments were not replicated. Thus, the role of pre-exposure to 17,20-BP on sensitivity to VT requires further study.

Extensive evidence from other species suggests that the effects of VT/VP on social behavior vary widely by species (Lema and Nevitt, 2004; Santangelo and Bass, 2006; Carneiro et al., 2003; Thompson and Walton, 2004; Lindeyer et al., 2015; Young et al., 1999; Pitkow et al., 2001) and/or by context (Ota et al., 1999; Lema, 2006; Goodson et al., 2009; Marler et al., 1999; Cooper et al., 2005). Although the current study does not provide evidence for context specificity in goldfish, this is likely due to a stress response during testing. Thus, future work should focus on reducing stress levels during pheromone exposure and testing to more accurately assess the effects of VT on courtship behaviors, and how these effects may be mediated by social context.

## ***Conclusions***

The current study provides compelling preliminary evidence for a mechanism by which VT could exert context-specific effects on goldfish social behavior, perhaps through context-dependent modulation of canonical and truncated VTR constructs. We found that both canonical and truncated VTR are likely translated into proteins that localize in overlapping distribution patterns throughout the forebrain and hindbrain. We also found that peripherally-injected VT inhibited approach to other males, and future work is needed to determine VT's effects in other social contexts. These results add to a growing body of research examining the species- and context-specific effects of VT and VP, two important, highly conserved neuropeptides that act in the SBN to influence social behavior across vertebrates. Ultimately, this study contributes to our knowledge of the mechanisms through which social context may modulate neuropeptide functioning, which is essential to understanding how vertebrates process social stimuli and produce unique and appropriate behavioral responses.



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## Supplementary figures

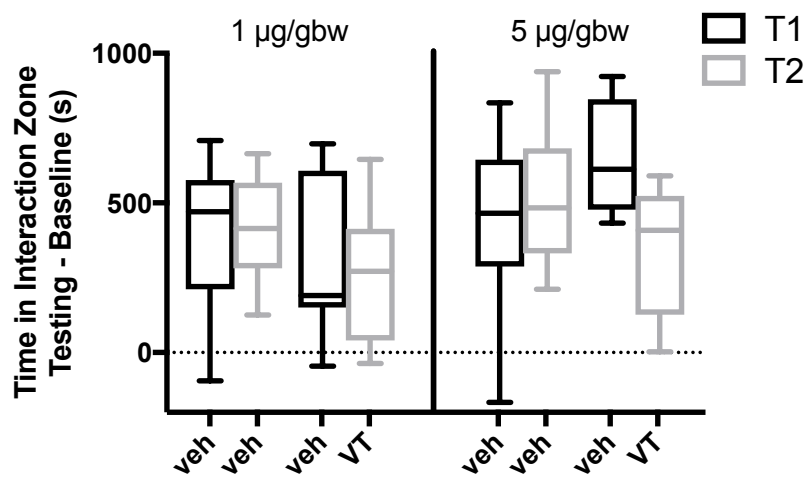


Figure S1. Approach to males at low and high VT doses in Experiment 1.

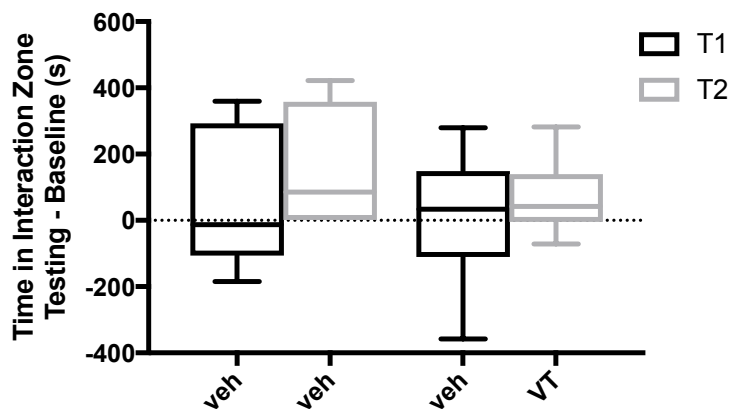


Figure S2. Approach to females following exposure to 17,20-BP in Experiment 2.

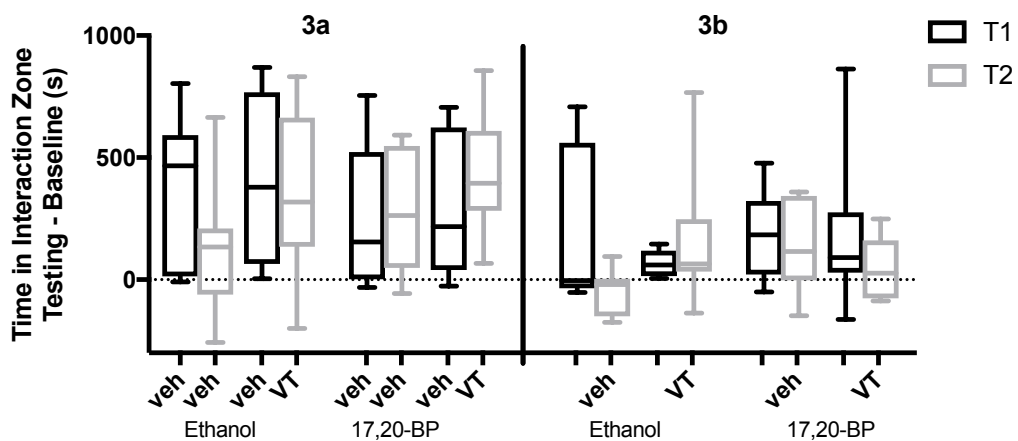


Figure S3. Approach to males following exposure to either 17,20-BP or ethanol in Experiment 3.